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Cloning and Expression of Genes for Dengue Virus Type-2 Encoded-Antigens for Rapid Diagnosis and Vaccine Development

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by

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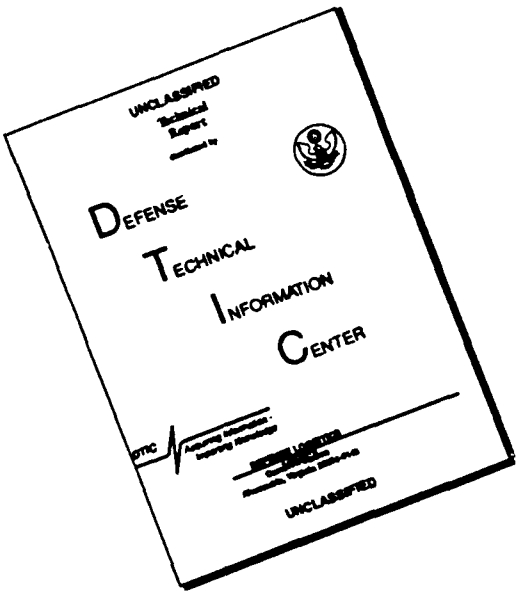

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1. Using a prokaryotic expression system, it was possible to map the antigenic determinant involved in neutralization of 3H5 monoclonal antibody.
2. A synthetic peptide containing the epitope elicits good neutralization antibodies with high titer in rabbits
3. Using a novel strategy, it was possible to produce truncated RNA molecules containing various lengths of 3'-terminal sequences of DEN-2 RNA with the same polarity and nucleotide sequence. The in vitro synthesized RNA could be labeled to a high specific activity with P-32.
4. Using radiolabeled RNA molecules, we could demonstrate the formation of a stable secondary structure containing the 3'-terminal sequences.

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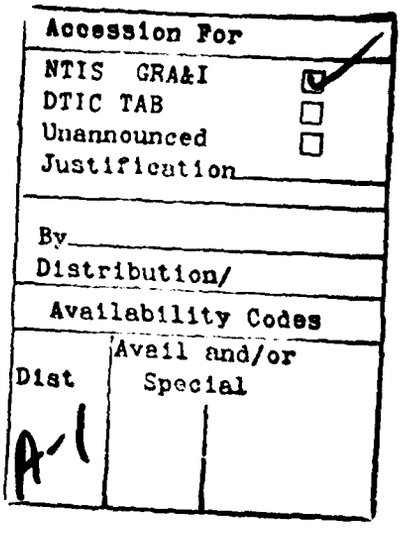
TABLE OF CONTENTS



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R Ramanathan 06/04/90

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3.0 INTRODUCTION

The family of flaviviridae consists of about 70 closely related enveloped viruses (Westaway et al., 1985) containing a single-stranded RNA of approximately 11 kb as their genome with a positive-stranded polarity (Russell et al., 1980). The 5'-end of the RNA has a type I cap structure, and a poly (A) track toward the 3'-end is absent.

Dengue, a human disease of paramount importance, is caused by dengue virus, a member of the flaviviridae Dengue viruses are of four distinct serotypes (DEN-1 to 4) and are transmitted to humans principally by Aedes aegypti mosquitos. In endemic areas of tropical Asia, apart from dengue fever (DF), a more severe form of the disease, dengue hemorrhagic fever (DHF), occurs in children, which could lead to dengue shock syndrome (DSS). Recently the pathogenesis of dengue was the subject of an excellent review by Halstead (1988).

The wide geographical occurrence of dengue infections combined with increasing number of epidemics in Central and South Americas and the Caribbean is a cause of major concern . An effective vaccine is not available to protect individuals against all four serotypes of DF. The major problem associated with dengue vaccine is that individuals having protection against one serotype are fully susceptible to infection with other DF serotypes. More often, the secondary infection with another serotype results in a serious form of the disease, DHF. Moreover, there are geographical heterogeneities in multiple dengue serotypes, as well as the genotypic variants of the same serotype (Trent et al., 1983; Repik et al., 1983; Kerschner et al., 1986; Walker et al., 1988). Using the techniques of RNA oligonucleotide finger printing and hybridizations with synthetic DNA probes, 15 genotypic variants for DEN-2, 7 for DEN-1 and 5 for DEN-3, have been characterized (Trent et al., 1983; Repik et al., 1983; Kerschner et al., 1986). Because of the rapid occurrence of variations in dengue viruses, there is a need to obtain the complete nucleotide sequence data of all the DEN serotypes and also of the important strains of the same serotype.

This would make it possible to relate protein structure to specified surface epitopes and facilitate the development of a recombinant vaccine.

Plaque reduction neutralization test (PRNT) is the most specific test for the identification of dengue virus serotypes. Gentry et al. (1982) have reported production of monoclonal antibodies directed against antigenic determinants of DEN-2 (New Guinea-C (NGS-C) strain. Both type specific and broadly cross-reactive antibodies were observed. 3H% was characterized to be a DEN-2 type-specific antibody with a neutralization titer of 1:3200, but having a low hemagglutination inhibition (HAI) titer (1:20). It also exhibited the highest RIA titer (1×10^6) which was 30 to 300-fold greater than the others in this group of type-specific monoclonal antibodies. These monoclonal antibodies have resolved at least three antigenic determinants on the virion that are functional in certain serological tests used to classify and identify flaviviruses: 1) virus type-specific determinant associated with strong virus neutralization but weak HAI, 2) a type-specific determinant associated with HAI, but not active in neutralization tests, 3) a cross-reactive determinant which is readily detected by HAI, but is also involved in low level cross-reactive neutralization. The type-specific monoclonal antibody 3H5 reacted by IFA with 11 other dengue 2 virus strains from various geographical areas, indicating that this antibody is serotype specific but not strain specific. Using a solid phase blocking RIA and rabbit anti-isotype antibody to block the binding of ^{125}I-labeled anti-mouse serum, 3H5 was found to be a IgG 1-secreting cell line (Gentry et al., 1982). No correlation was observed between neutralizing and antibody-dependent enhancement titers. 3H5 enhanced infection of only five of seven DEN-2 strains. The two DEN-2 strains with which 3H5 had low titers for enhancement of infection are PR159 and NGS-C (Halstead et al., 1984). Therefore, neutralization and ADE can be distinct virus-antibody interactions. Identification of these sites of the viral glycoprotein is important to produce recombinant vaccine. One approach to map these sites is to use monoclonal antibodies as reagents (Roehrig et al., 1982; Roehrig et al., 1983; Lubeck and Gerhard, 1982; Emini et al., 1982, Volk et al., 1982; Massey and Schochetman, 1981; Mathews and Roerig, 1984; Mehra et al., 1986). Using this approach, Heinz et al., presented a model for the antigenic structure of flavivirus

glycoprotein E consisting of variable and conserved epitopes (Heinz et al., 1983). A protein molecule consistes of a large number of different antigenically reactive sites recognized by antibodies or T cells (Todd et al., 1982). Various methods are available to determine the precise location of these sites (Benjamin et al., 1985; Berzofsky, 1985). The most commonly used approaches are to isolate and sequence neutralization escape variants, screening purified proteolytic fragments and screening collections of overlapping synthetic peptides. One approach using recombinant DNA techniques is to construct expression libraries containing fragments of the gene encoding the antigen using λgt 11 vector, and sequence the cDNA expression clone that reacts with the antibody. The DNA sequence encoding the epitope is attributed to sequences that are shared by multiple antibody-positive recombinant clone. We describe in this report another useful approach for mapping the epitope of a monoclonal antibody. First, an expression cDNA clone was constructed, from which a number of in-frame deletions spanning the antigen coding sequence were constructed to broadly map the epitope on the antigen molecule. The antigenic determinant is then precisely located by constructing a series of targeted deletions using polymerase chain reaction. Using this approach, we were able to define the neutralization epitope of a monoclonal antibody against the glycoprotein E of dengue virus type 2. The monoclonal antibody 3H5 chosen for epitope analysishas been shown in previous studies to have a significantly high neutralization titer (see above). In addition we show that a synthetic peptide was able to elicit neutralizing antibodies in rabbits as determined by PRNT. One limitation of this approach like other approaches is that topographically complex antigenic determinants involving noncontiguous sequences on the protein molecule could not be mapped.

In the second part of this progress report, we describe experimental evidence for the formation of a stable secondary structure at the 3'-end of DEN-2 RNA. The 3'-terminal sequences of approximately 100 nucleotides of flavivirus genomes have been suggested to have a highly conserved secondary structure, based on predictions of known nucleotide sequence data, and free energy calculations using computer programs. In order to test the existence of secondary structure in solution, we devised a strategy to generate truncated RNA molecules from about 0.3 to 1.4

kilobases in length in vitro, having the same polarity and nucleotide sequence as dengue virus type 2 (DEN-2) RNA (NEW Guinea-C strain). When these labeled RNA molecules were digested by RNase A, and analyzed by denaturing polyacrylamide gel electrophoresis, three protected fragments of 23, 28, and 33 nt in length were reproducibly obtained. To examine whether these RNase A-resistant fragments emerged from a stable secondary structure formed in solution consisting of 3' terminal sequences, hybridization of the resistant fragments to four chemically synthesized oligodeoxynucleotides, complementary to nt 1-24, 25-48, 49-72, and 73-96 from the 3'-terminus of DEN-2 RNA, followed by RNase H digestion were carried out. Two of the four oligodeoxynucleotides were sufficient to render all three RNase A-resistant fragments susceptible to RNase H digestion. From these data, it is clear that a stable secondary structure is formed in which the nucleotides 18- 62 from the 3'-terminus are very likely to be involved, and in addition, it is possible to deduce the RNase A cleavage sites. The potential use of these unique transcripts to identify the viral and/or host proteins which might interact at the 3'-terminus of DEN-2 RNA during initiation of replication is discussed.

4.0 Body of the Report

I. Expression of DEN-2 E protein and truncated polypeptides in E. coli and Immunological analysis

a) Rationale

One of the specific aims of the Contract proposal is to express the antigens coded by the dengue virus type 2 in order to understand their functions in replication, as well as in host immune response and pathogenesis. Heinz et al., (1983) showed that only those monoclonal antibodies which neutralized the virus in vitro also passively protected mice against lethal challenge with tick-borne encephalitis virus. Lobigs et al., (1987) defined the antigenic determinant involved in the neutralization of YF on E protein. The approach used was to select neutralization escape variants against two neutralizing monoclonal antibodies, and sequence the variants in the region of the genome encoding E and M. It was found that each of the variant resulted from a single nucleotide

change in the E region leading to a non-conservative amino acid substitution at position 71 or 72. Thus, the antigenic determinant (s) involved in the neutralization are localized in the E protein. Therefore, it was important to express E protein to analyze its antigenic determinants.

We chose pOTS expression vector for producing E protein of DEN-2 in E. coli because the synthesis of the antigen can be tightly controlled. The vector contains the strong λP_L promoter, and expression of any gene cloned under the control of this promoter can be induced with temperature. The physical map of the vector is shown in Fig. 1. The host is E. coli N5151 which produces thermo-labile λ repressor, which is inactivated at 42 C. The expression of nearly full-length E protein, or the truncated polypeptides were tested for their imuunoreactivity by Western blotting technique using the polyclonal hyper mouse ascitis fluid (HMAF), and the monoclonal 3H5 antibody. First, the epitope of 3H5 antibody on E protein was localized within a 180 amino acid residue region between two BamHI sites within the coding region of E protein. Subsequently, more targeted deletions within the 180 amino acid region were made using the polymerase chain reaction (PCR). Oligonucleotide primers were synthesized for use in PCR to amplify specific region , but to exclude a specific segment within 180 amino acid region. Using this approach, a series of targeted in-frame deletions were successfully made and tested for reactivity with both polyclonal HMAF, as well as the monoclonal 3H5 antibodies. The results of such analysis are presented below.

b) Experimental

1. Materials. Expression plasmid vector pOTS , *E.coli* strain MM294 [CI^+] and N5151 [CI^{857}] were kindly provided by Dr. Allan R. Shatzman [Smith Kline and French Labs, PA]. Plasmid pKT2.4 , contains the entire structural gene for C, prM, M and almost complete coding sequence of E glycoprotein of Dengue type 2 New Guinea 'C' strain, was cloned and sequenced in our laboratory (Irie et al., 1989). 3H5 monoclonal antibody and HMAF antibody against DEN- 2 virus were prepared and characterized as described previously (Brandt et al., 1967) and kindly provided by Drs. Robert Putnak and Mary K. Gentry, Walter Reed Army Institute of Research Washington, D.C. CV1 cells were grown at 37 C in DMEM supplemented with 10% fetal calf

serum. The New Guinea- C strain of DEN-2 virus was obtained from Walter Reed Army Institute of Research, Washington, D.C.

2. Construction of expression plasmids

Plasmid pOTS contains λP_L promoter for efficient transcription and λCII ribosome binding site. Unique BamHI restriction site has been engineered adjacent to the ribosome binding site (Fig. 1). The pOTS vector was digested with BamHI and blunted the ends by treatment with Klenow enzyme(large fragment of *E.coli* DNA polymerase I).This vector was used for genetic manipulation of E coding sequence. In general , the deletions were constructed by digesting E coding sequence with the appropriate restriction enzyme, and subsequently either removing the 3'- or filling in the 5'-cohesive ends. Digestion of pKT 2.4 plasmid (Irie et al., 1989) (Fig. 2) with PvuII and EcoRI released a DNA fragment which contains the most of E gene, followed by treatment with Klenow enzyme to blunt the ends, and ligation to pOTS vector were carried out. For expressing truncated E- polypeptides, pKT 2.4 plasmids were digested with restriction enzymes Tth111 I and EcoRI, ScaI and EcoRI, SphI and EcoRI, and partial NcoI, followed by EcoRI (Fig. 3). The DNA fragments encoding E-polypeptide with increasing NH_2-terminal truncation and a common COOH-terminal portion were made blunt ended, and subsequently ligated to pOTS vector. To generate COOH- terminal deletion mutants of E-polypeptide,the plasmid containing E coding sequence from restriction site Tth111 I to EcoRI was digested with BamHI restriction enzyme to remove a 506 base-pair DNA fragment [map position 1697 to 2203] (Fig. 2). The plasmid was then religated to create an internal deletion mutant of E- polypeptide at the COOH-terminal half of E protein. In all cases, the reading frame for the expression of the corresponding E- polypeptide was kept in-frame, and was verified by DNA sequence analysis. The plasmids derived from pOTS were used for transformation into *E. coli* strain MM 294 CI^+ to maintain repression of λP_L promotor during analysis of the constructs. Screening was carried out by agarose gel electrophoresis of appropiate restriction digests. Recombinant plasmids containing

E-polypeptide coding sequences in proper orientation were then used for transformation into *E. coli* strain N5151 CI^{857} which expresses thermolabile λ repressor.

3. Expression of E- polypeptide in E. coli

E. coli N5151 cells containing recombinant plasmids were induced as described for pKC 30 and its derivative [5]. Briefly, the bacterial cells were grown at 32 C to 1.0 A_{650} in LB medium containing 100 μg/ml ampicillin, then the temperature was shifted to 42 C by adding equal volume of LB medium, prewarmed to 65 C, and the culture was incubated at 42 C for another 1 h. The cells were harvested by centrifugation and washed once with TES buffer [Tris- EDTA- NaCl buffer, pH 7.5].

4. Construction of plasmids with truncated E coding sequences by Polymerase Chain Reaction.

It is generally accepted notion that the entire surface of a protein is antigenic (Benjamin et al., 1984), and the antigenic determinant involved in neutralization is likely to be a hydrophilic domain exposed to the surface of the protein. Therefore, deletion of a specific hydrophilic domain within a broadly defined region consisting of neutralizing antigenic determinant was carried out by PCR, followed by antibody screening in a Western blot. Synthetic oligonucleotide primers for deletion by PCR were chosen from surface regions predicted from the hydropathy plot of Kyte and Doolittle [1982; see Fig. 5]. The BamHI restriction fragment from map position 1697 to 2203 of DEN2 sequence corresponding to amino acid residues 254 - 423 of E- polypeptide (Irie et al., 1989) was plotted and scanned amino acid sequence for residues which have a high probability of being exposed on the surface of the molecule. Based on these data, seven different peptide segments contain a stretch of hydrophilic amino acid residues. The synthetic oligonucleotide primers (#1-6 in Fig. 4) were chosen from the nucleotide sequences of hydrophobic amino acid residues downstream from the nucleotide sequences that coded for hydrophilic amino acid residues. The

primer#1 5'- CCAAGAAGGGGCCATGCACACAGCA -3' maps at position 1701 of DEN2 genome. The choice of this 5'-terminal primer for PCR was based on the fact that when the amplified DNA fragment was ligated to expression plasmid pOTS, it would regenerate the BamHI

restriction site at the map position 1697 in the proper orientation of the insert. Primers # 2 to #6 were chosen as 3'-terminal primers for PCR from the complementary strand of DNA , and have the following sequences.

primer#2 3'- TACAGTATGAGATACACGTGTCCTT - 5' begin at map position 1824

primer#3 3'- CCAGCGGACTAATGTCAGTTGGGTT - 5' begin at map position 1921

primer#4 3'- ATGTAGTATCCTCATCTCGGCCCTG - 5' begin at map position 2064

primer#5 3'- TCATTTGGCCGATAGAACT - 5' begin at map position 2121

primer#6 3'- CCACTGTGTCGAACCCTAG - 5' begin at map position 2182

By using a mixture of two primers (primer#1 being constant and the second primer is chosen from # 2-#6), and BamHI restriction fragment from E coding sequence as template for PCR, a series of amplified DNA fragments which would include specific in-frame deletions can be generated. In PCR reaction with primer#1 and #2, it should generate DNA fragment of 148 base pairs fragment (map position 1701 to 1849). With primer#1 and #3, #1 and #4, #1 and #5, and #1 and #6, it should generate DNA fragments of 245 (1701 to 1946), 388 (1701 to 2089), 439 (1701 to 2140) and 500 (1701 to 2201) base pairs respectively. The last amino acid of each PCR generated DNA fragment (amino acid residue 423), when ligated to expression vector, was arbitrarily changed from the original glycine to arginine (GGA to AGA or CGA). DNA from each PCR reaction was ligated to pOTS vector. Screening of transformants were carried out by BamHI digestion for proper orientation, and the recombinants were then transformed into N5151 CI^{857} for expression. All constructs were sequenced by using synthetic oligonucleotides primers homologous to sequence upstream and downstream from both BamHI restriction sites, to determine the nucleotide sequence to confirm the predicted reading frame and reliability of PCR reaction.

5. DNA amplification by Polymerase Chain Reaction

Amplifications using *Taq* Polymerase were performed according to procedure described by the manufacturer (Gene Amp. Perkin - Elmer Cetus). Briefly, 506 bp DNA fragment from E protein

coding sequence was used as a target sequence. The DNA was amplified in a 100 µl reaction volume containing 100 ng of template, 4 unit of *Taq* polymerase, 125 mM dNTP, 1 µM each of primer. The reactions were performed for 25 cycles. The amplified DNA were fractionated by 1.5% agarose gel electrophoresis, and DNA was purified from agarose gel by glass powder elution (Vogelstein and Gillespie, 1979).

6. Polyacrylamide gel electrophoresis and Western Blot

Total bacterial pellets were resuspended in 300 µl of Laemmli sample buffer [Laemmli, 1970] and heated to 100 C for 5 min. The suspensions were subjected to electrophoresis through 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [SDS]. Total bacterial lysates [15 µl] were loaded in 5 mm. slots. Proteins were then transferred to nitrocellulose in 25 mM Tris- 150 mM Glycine, pH 8.3- 20% methanol and 1%SDS at 200 mA for 4 h. Blots were blocked for 30 min. in 22% nonfat dry milk / 0.9% NaCl/ 10 mM Tris HCl, pH 7.5. After washing with 50 mM Tris HCl, pH 7.5/ 200 mM NaCl [TBS], the blots were then incubated sequentially with 1:500 3H5 monoclonal antibody or 1:500 hyperimmune mouse ascites fluid, goat anti-mouse IgG conjugated with alkaline phosphatase [Hyclone, Logan Utah], and the substrate 5- bromo- 4- chloro- 3- indolyl phosphate and nitroblue tetrazolium. Blots were washed between incubations with TBS 3 times, 10 min each.When color had developed sufficiently, the reaction was stopped by adding stop buffer [20 mM Tris HCl- 5 mM EDTA].

7. Peptide synthesis and coupling

Peptide, Q-L-K-L-N-W-F-K-K-G-S-S, corresponding to amino acids 386 to 397 of E protein of Dengue virus type 2 with an additional cysteine residue attached to the NH_2-terminus, was synthesized by solid phase synthesis on an automated peptide synthesizer (Sam Two, BioSearch). Synthetic peptide was purified by high performance liquid chromatography. The coupling reagent m-maleimidobenzoylsulfosuccinimide ester (Pierce Chemical Co., Rockford Ill.) was used to attach the peptide, via N-terminal cysteine residue, to Keyhole limpet hemocyanin (Sigma)

according to the procedure of Liu and co-workers (1979); coupled peptide was stored at -20 C for use.

8. Antibody Production

Antiserum to synthetic peptide-KLH conjugate was obtained from 10 week-old male New Zealand white rabbit. The animal was first inoculated intramuscularly with 200 μg peptide-KLH conjugate emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). On day 14, 100 μg peptide-KLH conjugate in 0.5 ml PBS and 0.5 ml Freund incomplete adjuvant was injected subcutaneously at multiple sites along the back. The rabbit was reinoculated four times at weekly intervals with the same amount used in the procedure for second inoculation. The rabbit was bled from ear vein 10 days after last injection.

9. ELISA

Reactivity of antisera with synthetic peptide was measured by a modification of the enzyme-linked immunosorbent assay (ELISA) protocol described by Engvall (1980). Microtiter plates (Corning, New York) were coated with 50 μl of synthetic peptide (20 μg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 37C. The antigen coated wells were washed 3 times with 0.85% NaCl containing 0.05% Tween 20. After washing, the plates were blocked with 5% BSA in PBS-Tween 20 (100 μl/well for 1 h. at 37C. Plates were then incubated sequentially with 50 μl/well of appropiate dilution of antiserum in PBS-Tween 20 containing 1% BSA, goat anti-rabbit antibody conjugated to horseradish peroxidase (Hyclone, Logan, Utah.), and the substrate orthophenylenediamine(Sigma). Plates were washed between incubations with 0.05% Tween 20 in 0.85% NaCl. The final enzyme reaction was stopped with 2N H_2SO_4, and the color change was measured at 492 nm on a Titertek Multiskan plate reader (Flow Laboratories, McLean Virginia.)

10. Neutralization Test

Antiserum was inactivated at 56 C for 30 min. and serial 2-fold dilutions beginning at 1:10 were made. Appropriate dilutions of antiserum were incubated with 100 pfu of virus for 2 h. at room temperature. These mixtures were then assayed by plaque reduction neutralization test [Russell and Nisaluk, 1967]. Briefly, monolayers of CV1 cells were prepared by seeding $0.5x10^6$ cells in each well of 6-well tissue culture plates [Costar, Cambridge Mass.]. After overnight incubation, the growth medium was removed and each plate was inoculated with 0.3 ml antiserum - virus mixture. Adsorption of the virus inocula was carried out for 1 h at room temperature. After washing the monolayer of cells with MEM without serum, it was overlaid with 3 ml of MEM containing 0.9% agar. After the agar had solidified, the plates were incubated at 37 C in 5% CO_2 atmosphere. And the second overlay containing neutral red was added on the 6^{th} day. Plaques were counted on the 7^{th} day. Titers from plaque reduction neutralization test were expressed as mean percent plaque reduction.

11. Immunoprecipitation Assays

CV1 cells in T75 flasks were infected with DEN2 New Guinea C strain at approximately 1 pfu/cell. After 72 h post-infection, [^{35}S] methionine (10 µCi / ml) was added with methionine deficient medium for 3 h. The cells were disrupted by scraping, and cell pellets were obtained by low speed centrifugation. Cells were solubilized in RIPA buffer, containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% DOC, 0.5% NP40 and 0.1 mM PMSF, and sonicated for 1 min. The lysates were centrifuged at 100,000 x g for 1 h. Supernatants were mixed with appropriate antibodies and protein A agarose beads (Genzyme Corp., Boston, Mass.). The precipitates were washed, dissociated by boiling in sample buffer (Laemmli, 1970), and analysed by 10% SDS-PAGE. Gels were fixed, stained and processed for fluorography .

c) Results and Discussion

1. Expression of E polypeptide in E. coli

The approach which was taken to map the antigenic determinants of envelope glycoprotein E of Dengue virus type 2 was first to express E protein in E. coli using the pOTS expression vector

system. Next, several deletion clones were constructed in the NH2- terminal, COOH-terminal, or internal regions such that the open reading frame was maintained, and analyzed for their expression in E. coli. The parent and the truncated polypeptides of E protein were tested for their reactivities with the monoclonal antibody 3H5 to map the location of a neutralization determinant of the E protein recognized by the antibody. The NH2-terminal deletions of E protein were constructed in pOTS expression plasmid, by using the restriction sites for Tth111I (pTT 15), Sca I pTT 20), Sph I (pTT 25), and NcoI (pTT 35) present in the gene. The internal deletion construct of E polypeptide was also constructed by removing the region encoding about 180 amino acid residues flanking the two Bam HI restriction sites. This mutant contruct (pTT 30) should give rise to E polypeptide that lacks amino acid residues 255 to 422 in the viscinity of the COOH- terminal portion of E polypeptide. The level of expression of E polypeptides in E. coli was rather low from many of the plasmid constructs. However, the pTth construct which contains the coding region of E polypeptide from amino acid residues 22 to 469, gave rise to a level of the gene product that was high enough to be visualized directly by Coomassie blue staining of the polyacrylamide gel (data not shown). The low level of expression of other polypeptides might be due to their instability, or toxicity or both. The synthesis of these polypeptides could be detected only by Western blotting technique using HMAF or 3H5 monoclonal antibody (Figs.7 and 8). The sizes of some of these polypeptides correlated well with their predicted values. The expected full-length polypeptide from each plasmid, as well as shorter fragments that may have arisen by proteolysis or incomplete translation were seen, except for plasmid construct pTT 10. This plasmid contains at the N-terminus of E protein extra seven hydrophobic amino acids of the preceding ORF in the COOH-terminal portion of M protein. This hydrophobic stretch of residues might render the polypeptide sensitive to bacterial proteolytic enzymes. The pTT 10 plasmid expressed a protein of 46 kDa, and major product of 36 kDa, which is probably a degradation product of full-length protein. The reactivity of HMAF and 3H5 monoclonal antibody to various truncated derivatives of E polypeptide was examined by western blot analysis (Figs.6a and 6b). Polyclonal antibody (HMAF) reacts with all E polypeptides. Monoclonal antibody 3H5 reacted with all

mutant polypeptides with NH_2-terminal deletions, but did not react with the truncated polypeptide (lacking amino acid residues 255 to 422) produced from the internal deletion construct which lacked the 506 base pair region between the two BamHI sites (Fig. 6b. lane 6). From these data we can conclude that the antigenic determinant for 3H5 monoclonal antibody is localized within 167 amino acid residues (between amino acid residues 255 to 422) (Fig. 4).

2. Precise mapping of the epitope of 3H5 monoclonal antibody

In order to map the epitope of 3H5 monoclonal antibody more precisely, we developed a strategy by constructing a series of targeted deletions within this region of 167 amino acids, using polymerase chain reaction (PCR). It is generally accepted notion that most, if not all, of the antigenic determinants reactive with antibodies are exposed to the surface of the antigen molecule. The hydropathy plot of the region encoding the 3H5 epitope is shown in Fig. 5, and the predicted surface residues (hydrophilic amino acid stretch) are indicated. The hydropathy profile predicts three major linear surface regions (10 or more residues in length) at amino acid residues 285-298, 358-374, and 386-397, and three minor regions (five or more residues in length) at amino acid residues 325-333, 341-347 and 404-410. A set of deoxyoligonucleotides from #1 to #6 were synthesized corresponding to the regions shown in Fig. 4. PCR amplification was carried out using the primer #1 and any one of the primers #2-#6 on the 506 bp region between the two BamHI sites as the template. Each amplified DNA fragment from PCR reaction was ligated to the blunt-ended BamHI sites of pTth expression plasmid, which gave the highest levels of nearly full-length E protein. When the primer pairs of #1 and #2, #1 and 3, #1 and #4, #1 and #5, #1 and #6 were used in PCR, DNA fragments of sizes 148 bp (encoding amino acid residues 255-304 which included a hydrophilic region A between 285-298), 245 bp (including the hydrophilic region A plus two minor regions of 325-333 and 341-347), 388 bp (which included the previous regions plus a major hydrophilic domain of 358-374), 439 bp all predicted linear surface regions except the minor region of 404-410), and 500 bp (encoding the entire region between the two BamHI sites), respectively, were generated. DNA from each PCR was ligated to pTT15 expression plasmid as

described in materials and methods, and the polypeptides that were produced were analyzed by immunoblots.

3. Reactivity of truncated polypeptides generated by PCR

The lysates of transformants obtained from deletion constructs were tested by Western blot for the reactivity of the truncated E polypeptides with polyclonal HMAF. The sizes of the expressed polypeptides agreed well with the predicted values. The smallest size is 34 kDa which was expressed from amino acid residues 22 to 305 fused to the COOH-terminal region from amino acid residues 423-469. The other clones expressed truncated polypeptides of 38 kDa (34 kDa plus residues 306-357), 41 kDa (38 kDa plus residues 358-385), 43 kDa (41 kDa plus residues 386-397), and 44 kDa (41 kDa plus residues 398-423) (Fig. 4), encoded from the amplified DNA fragments of increasing length were cloned in-frame. When these truncated polypeptides were tested for their reactivity with 3H5 monoclonal antibody, only the truncated polypeptides of 43 and 44 kDa reacted with 3H5. The difference between 41 kDa and 43 kDa polypeptide is the hydrophilic region containing residues, QLKLNWFKKGSS (386-397) (see Fig. 4). When this stretch of residues was included, the expressed polypeptide reacted with 3H5. This stretch of twelve amino acids is encoded by DNA sequences between primer #4 and primer #5. The 44 kDa polypeptide also reacted with 3H5 antibody as expected due to the presence of this hydrophilic region.

4. Reactivity of anti-peptide antibody with synthetic peptide antigen.

We synthesized a peptide of twelve amino acid residues corresponding to the identified epitope of 3H5 monoclonal antibody, and used this peptide to immunize rabbit. The reactivity of the immune sera with the peptide was monitored by ELISA . Anti- peptide antibody reacted specifically with the peptide at the dilution of 1:10,000, when compared with preimmune sera (Fig.8b). The reactivity of 3H5 monoclonal antibody to peptide also showed specificity when compared with unrelated monoclonal antibodies 3E9 and A16, which are monoclonal antibodies against DEN-2 NS1 protein and Herpes virus glycoprotein, respectively (Fig.4).

5. Immunoprecipitation of E Proteinby anti-peptide antibody

CV1 cells were infected with DEN-2 virus, and labeled the proteins using ^{35}S methionine. Immunoprecipitation of labeled proteins from the infected cells, followed by SDS/PAGE indicated that anti-peptide antibody recognized a protein with a mobility in gel similar to that of gp 60 immunoprecipitated with either HMAF or 3H5 monoclonal antibody (data not shown).

6. Plaque Reduction Neutralization Test using anti-peptide Antisera

Rabbit anti serum which reacted with peptide in ELISA was used for plaque reduction neutralization test. Anti-peptide antibody was found to neutralize the infectivity of DEN-2 virus (Table 1). The titer of neutralization was expressed as mean percent plaque reduction . The 50% neutralizing capacity was observed at the titration of 1:40.

II. Detection of stable secondary structure at the 3'-terminus of dengue virus type 2 RNA

a) Rationale

The 3' terminal sequences of approximately 100 nucleotides of flavivirus genomes have been suggested to have a highly conserved secondary structure, based on predictions from the known nucleotide sequence data, and free energy calculations using computer programs (Tinoco et al., 1973). Two lines of experimental evidence suggested that such secondary structures could exist in solution. First, Brinton et al. (1986) found that nucleotides within the putative region of secondary structure were partially resistant to ribonuclease. Second, Hahn et al., (1987) reported the isolation of rare cDNA clones of DEN-2 RNA (S1/candidate vaccine strain of PR-159 isolate) as well as that of MVE RNA, which could have arisen by self-priming of the 3'-terminal base-paired region during reverse transcription. In order to test the existence of secondary structure in solution, we devised a strategy to generate truncated RNA molecules from about 0.3 to 1.4 kilobases in length in vitro, having the same polarity and nucleotide sequence as dengue virus type

2 (DEN-2) RNA (NEW Guinea-C strain). When these labeled RNA molecules were digested by RNase A, and analyzed by denaturing polyacrylamide gel electrophoresis, three protected fragments of 23, 28, and 33 nt in length were reproducibly obtained. To examine whether these RNase A-resistant fragments emerged from a stable secondary structure formed in solution consisting of 3' terminal sequences, hybridization of the RNase A-resistant fragments to four chemically synthesized oligodeoxynucleotides, complementary to nt 1-24, 25-48, 49-72, and 73-96 from the 3'-terminus of DEN-2 RNA, followed by RNase H digestion were carried out. Two of the four oligodeoxynucleotides were sufficient to render all three RNase A-resistant fragments susceptible to RNase H digestion. From these data, it is clear that a stable secondary structure is formed in which the nucleotides 18- 62 from the 3'-terminus are very likely to be involved, and in addition, it is possible to deduce the RNase A cleavage sites. The potential use of these unique transcripts to identify the viral and/or host proteins which might interact at the 3'-terminus of DEN-2 RNA during initiation of replication is discussed.

b) Backround and Significance

A model was proposed for the possible secondary structure in the 3'-terminal 87 nucleotides of YF RNA (Rice et al., 1985). Since then, a number of flavivirus genomes have been cloned and sequenced, such as West Nile virus (Castle et al., 1985; 1986; Wengler et al., 1986), DEN-4 (Zhao et al., 1986; Mackow et al., 1987), three distinct isolates of DEN-2 (Hahn et al., 1988; Duebel et al., 1986;1988; Yaegashi et al., 1986; Irie et al., 1989), JE (Takegami et al., 1986; Sumiyoshi et al., 1987), and Kunjin virus (Coia et al., 1988). These sequence data support the general structural features of the flavivirus genome mentioned above.

In the present study, we tested the formation of such stable secondary structure in solution, by devising a novel method to generate radiolabeled transcripts of high specific activity in vitro, which contained authentic 3'-terminal sequences of DEN-2 RNA (NGS-C strain). By digesting these labeled transcripts with RNase A, three protected fragments of 23-33 nt in length were obtained. These RNase A-resistant fragments were mapped within the 3'-terminal 96 nucleotide

region by analyzing their distinct susceptibilities to RNase H digestion, subsequent to the formation of RNA:DNA hybrids with synthetic oligodeoxynucleotides of known sequence. These results strongly support that such stable secondary structures are indeed formed in solution. From these data it was possible to deduce the RNase A-sensitive sites of such a secondary structure in solution.

c) Experimental

(1) Plasmid constructs

The cDNA clone containing the 3'-terminal sequence of DEN-2 (NGS-C strain) was obtained by PCR as reported previously (Irie et al., 1989). Briefly, a cDNA library prepared from DEN-2 RNA which was tailed with poly (A) using Escherichia coli poly (A) polymerase (Sippel, 1973) was used for amplification by PCR (Saike et al., 1988. The oligodeoxynucleotides GGACAAGTTGGTACCTATGG (representing nucleotide number 9373-9392 of DEN-2 genome; see Irie et al., 1989) and AGAACCTGTTGATTCAACACACC complementary to the 3'-terminal sequence of DEN-2 genome were used as primers for amplification by Taq polymerase. The amplified DNA after a total of 25 cycles of denaturation, annealing and DNA synthesis was digested with KpnI and then purified by electrophoresis on an agarose gel. For cloning, pGEM7Zf$^+$ vector (Promega, Wisconsin) containing multiple cloning sites, as well as SP6 and T7 promoters was digested with XbaI, blunt-ended by treatment with PolIk (Maniatis et al., 1982), and then digested with KpnI (Fig. 9). The vector DNA was subsequently dephosphorylated at the 5'-termini. Ligation and cloning of the PCR-amplified DNA into the vector gave rise to several colonies which were screened for the presence of XbaI and KpnI sites. Several independent clones having these sites with the correct orientation were sequenced at their termini (up to about 120 nucleotides). The sequence data were found to be identical in this region to those reported previously (Irie et al., 1989).

Subclones from the parent 1.4 kb cDNA clone which all had identical 3'-terminal DEN-2 sequences with varying lengths toward the 5'-end were prepared by digesting the parent clone at the SacI site (Fig. 9), and treating with E. coli exonuclease III, followed by S1 nuclease (Henikoff, 1984). The end points of all deletions were verified by DNA sequence analysis.

(2) In vitro transcription

In order to get transcripts having 3'-terminal sequence identical to the DEN-2 genomic RNA, the parent clone or a subclone was digested with S1 nuclease. Labeled transcripts of high specific activity from this linearized plasmid templates were prepared by the method of Melton et al. (1984). The in vitro transcription was carried out in 25 µl reaction volume containing 40 mM Tris.HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 0.05 mM each of GTP, CTP, UTP, 0.5 mM ATP, one unit of Inhibit-ACE (5 Prime-3 Prime, Inc.,West Chester, PA), 1 mM $m^7G(5')ppp(5')G$, 100 µg bovine serum albumin, 10µCi each of (α-^{32}P) GTP, CTP, UTP (400 Ci/mmole) and two units of SP6 RNA polymerase. The reaction mixture was incubated at 40°C for 60 min. DNase I (1 unit) was then added and incubated further for 15 min. at 37° C. The reaction mixture was extracted with phenol: $CHCl_3$ and the unincorporated nucleotides were removed on Biospin-101 column (BioRad). The labeled transcript was extracted once with phenol:$CHCl_3$ and precipitated with ethanol and stored as small aliquots at -70°C.

(3) RNase A and T1 digestion

The (^{32}P) labeled transcripts ($5x10^4$ to 10^5 cpm) were digested with pancreatic RNase A (3µg) in 50µl reaction volume containing either 0.01X SSC or 2X SSC (1X = 0.015M Na-citrate and 0.15M NaCl) pH 7.0 at 37°C for 60 min. RNase A was removed by proteinase K (3µg) digestion in 1%SDS at 37°C for 15 min, followed by phenol:$CHCl_3$ extraction and ethanol precipitation in the presence of E.coli tRNA (3µg) added as a carrier. The labeled RNA was collected by centrifugation, and the pelletted RNA was finally dissolved in a small volume of 7M urea/Tris-borate/EDTA buffer, pH 8.3, containing bromophenol blue dye, heated at 90°C for 3

min. and loaded on a denaturing 20% polyacrylamide gel. The labeled transcript was digested with RNase T1 (1 unit) in 50 µl reaction mixture containing either 0.01 X SSC or 2X SSC as described above for RNase A digestion.

(4) RNase H digestion

The RNase A digested products were hybridized to the following oligodeoxynucleotides (24 mers): 1) AGAACCTGTTGATTCAACAGCACC, 2) ATTCCATTTTCTGGCGTTCTGTGC, 3) CTGGAATGATGCTGAGGAGACAGC , and 4) AGGATCTCTGGTCTTTCCCAGCGT, which are complementary to nt 1-24, 25-48, 49-72, and 73-96 of DEN-2 RNA, respectively, (the 3'-terminal nt as 1). The hybridization was done in 10 µl reaction volume containing 25mM Tris-HCl, pH 7.5, 300mM KCl, 2mM DTT, 1mM EDTA, 6mM $MgCl_2$ at 90°C for 3 min., followed by quick chilling in ice for 5 min. RNase H (1unit) was added and incubated at 37°C for 60 min. The reaction was stopped by the addition of stop buffer containing formamide (80%)-bromophenol blue dye. The samples were heated to 90°C for 3 min. and loaded onto a 20% sequencing gel.

(5) Polyacrylamide gel elecrtophoresis

The RNase A digested products were fractionated by electrophoresis on 20% polyacrylamide gels using Tris.HCl/borate/EDTA/7M urea buffer system at 1200V until the bromophenol dye reached 21cm. The gel was covered with plastic wrap and exposed directly to Kodak XAR-5 film at -70°C.

d) Results and Discussion

(1) Generation of transcripts containing the 3'-terminal sequences of DEN-2 RNA

Sequence analyses of a number of flavivirus genomes reveal that there is a highly conserved feature of secondary structure of the viral RNA at the 3'-terminus. To examine the potential of the 3'-terminal sequences to form secondary structure in solution, we devised a

strategy to generate truncated RNA molecules having the authentic 3'-terminus of DEN-2 RNA, differing only in their lengths at their 5'-end. The parent plasmid contains an insert of 1339 bp corresponding to the 3'-terminal sequence of DEN-2 RNA cloned between XbaI and KpnI sites of pGEM 7Zf$^+$ vector (Promega Biotec) (Fig. 9). Several subclones were generated from this parent plasmid by sequential digestion with E. coli exonuclease III and S1 nuclease (Henikoff, 1984) as described under MATERIALS AND METHODS. The end points of deletion in these subclones were determined by sequence analysis (Sanger et al., 1977). Two cDNA clones containing the 3'-terminal sequences of 292 bp, and 1339 bp were linearized with XbaI, followed by digestion with S1 nuclease (Fig. 9). They were then used as templates for in vitro transcription using SP6 RNA polymerase in the presence of labeled rNTPs to give rise to transcripts having the same polarity, as well as the 3'-terminal sequence as DEN-2 RNA, differeing only in their lengths.

(2) Protection against RNAse A digestion

To investigate whether labeled transcripts could form stable secondary structure in solution, digestion by RNase A was carried out under two different salt concentrations. Since double-stranded regions of RNA are resistant and single-stranded regions sensitive to RNase digestion, any secondary structure formation in RNA could be visualized as RNase-protected fragments by electrophoresis on polyacrylamide/urea gels followed by autoradiography. Fig. 10 shows that when labeled transcripts of 292 and 1339 bases in lengths were digested by RNase A in a buffer containing 1.5 mM NaCl and 0.15 mM sodium citrate (0.01 x SSC), or 300 mM NaCl and 30 mM sodium citrate (2 x SSC), some common RNase- resistant fragments were obtained. The sizes of the protected fragments were estimated to be within two clusters, the first between 23 and 33, the second between 1-15 nt long (Fig. 10A and 10B), by comparison with the ladder of 3'-labeled tRNA hydrolyzed by alkali (not shown). The fragments indicated by arrows in the first cluster were the predominant species, and their sizes were estimated to be about 33, 28, and 23 nt in length (Fig. 10A and 10B). Other RNase A-protected fragments produced in the digest of 1339 nt transcript were not present in the digest of 292 nt transcript in the first cluster, suggesting that these could have been produced from the region not shared by the two transcripts (between

292 and 1339 nt). interestingly, under the conditions of RNase digestion in low ionic strength buffer (0.01 X SSC), only the 33 nt fragment was produced as the RNase-resistant fragment (Fig. 10, lane 5).

Next, the question whether these RNase A-protected fragments resulted specifically from the transcripts containing the 3'-terminal sequences of DEN-2 RNA was addressed. A control transcript from the region of 1-550 nucleotides at the 5'-end of DEN-2 RNA was digested with RNase A under the same conditions of high ionic strength. Fig. 10B shows that the pattern of RNase-protected fragments (lane 4) was quite different from that of 3'-terminal transcript (lane 2).

(3) Mapping the location of RNase A-resistant fragments by annealing with synthetic oligodeoxynucleotides, followed by RNAse H digestion

To investigate further whether these RNase-protected fragments contained sequences from the 3'-terminal region of DEN-2 RNA , four oligodeoxynucleotides were synthesized complementary to the first 96 nucleotides of DEN-2 RNA. Subsequent to RNase A digestion of the two transcripts containing the 3'-terminal sequences, the RNase-protected fragments were annealed to each of the four oligodeoxynucleotides, and the annealed mixtures were digested with RNase H. Since RNase H digests only the RNA of the RNA:DNA hybrid, this experiment could reveal the specificity and location of the protected fragments. The results of RNase A, followed by RNase H digestion shown in Fig. 11 indicate that oligodeoxynucleotides #1 and #4 did not have any effect (lanes 3 and 6 in Fig. 11 A & 11 B). However, when oligomer 2 was annealed, 33 and 28 nt long protected fragments became sensitive to RNase H (Fig. 11, lane 4, A and B), indicating that these protected fragments resulted from the region of transcript corresponding to nt 25-48 from the 3'-terminus of DEN-2 RNA. Similarly, when oligodeoxynucleotide #3 was annealed, RNase-A protected fragments of 28 and 23 nt in length became sensitive to RNase H digestion, suggesting that these protected fragments contained sequences complementary to nt 49-72 from the 3'-terminus of DEN-2 RNA.

4) Evidence for a stable stem-loop structure at the 3'-terminus of DEN-2 RNA

The data presented in Figs. 10 and 11 support the presence of a stable secondary structure predicted from the primary sequence data and free energy calculations (Tinoco et al., 1973) for a number of flavivirus RNA molecules (Rice et al., 1985; Castle et al., 1985; Wengler et al., 1986; Zhao et al., 1986; Takegami et al., 1986; Sumiyoshi et al., 1987; Mackow et al., 1987; Hahn et al., 1988; Duebel et al., 1988; Coia et al., 1988; Irie et al., 1989). Fig. 12 A shows the stem loop structure proposed for DEN-2 RNA (Hahn et al., 1988). In order to test whether any such structure could be detected in solution, labeled transcripts were analyzed by RNAse A digestion. The RNase-resistant fragments were further analyzed to map their location in the putative stem-loop structure for the 3'-terminal sequence of DEN-2 RNA by annealing with a specific synthetic oligodeoxynucleotide, followed by RNase H digestion. None of the three fragments having sizes of about 33, 28, and 23 nt that were produced as RNase A-resistant fragments became sensitive to RNase H digestion when oligomers #1 and 4 were annealed indicating that their location mapped outside of the region covered by these oligomers (Fig. 12 A & B). When oligomer # 2 was annealed to the mixture of RNase A-resistant oligoribonucleotide fraction, and the mixture was digested by RNase H, it was found that fragments of 28, and 33 nt in length were susceptible to RNase H, whereas annealing of oligomer # 3 made a 23 and a 28 nt fragment sensitive to RNase H. If the putative cleavage sites of RNase A are as indicated by arrows (Fig. 12A), then cleavages occurring at sites 1 and 4 would give rise to a 33 nt fragment. Similarly, cleavages at 1 and 3, or 2 and 4 would give to a 28 nt fragment. Annealing of oligomer # 2 would make both these fragments susceptible to RNase H digestion. Additional fragments were produced subsequent to RNase H digestion (as shown by arrowheads in Fig. 11 A and B in lane 4) possibly due overlapping fragments generated from RNase A-sensitive sites at 3 and 5 (see below, and in Fig. 12B). Cleavages by RNase A occurring at sites 4 and 5 would be expected to produce a fragment of 23 nt in length, which upon annealing to oligodexynucleotide # 3 would make it susceptible to RNase H digestion. The data shown in Figs. 11A and 11B are consistent with this model of secondary structure for 3'-terminus of DEN-2 RNA. Our results support the existence of base-paired region and a general loop structure between nucleotides 18-62, because

long RNase A-protected fragments were generated from this region. However, the region between nucleotides 63-96 did not give any significant protection under our conditions of RNase A digestion. This is supported by the pattern obtained by RNase T1 digestion which generated major fragments of 16, 17, 28, and 34 nt fragments (Fig. 10C and Fig. 12A). This highly conserved secondary structure has been proposed to be important for replication of viral RNA (Hahn et al., 1987; Brinton et al., 1986; Takegami et al., 1986). Therefore, it will be interesting to test whether such a secondary structure is stabilized by binding of proteins in vivo. Our method to generate labeled transcripts having the same polarity and sequences at the 3'-terminus of DEN-2 RNA might be useful for such studies.

5. 0 Figure Legends

Fig. 1 Physical map of pOTS expression vector

The vector contains prokaryotic origin of replication (Ori), ampicillin resistance gene (amp^r), strong λPL promoter (P_L), ribosome binding site (Shine-Dalgarno sequence-SD), translational initiator codon as part of the unique BamHI site, multiple cloning sites, and transcriptional terminator (T_O) downstream. The arrow indicates the direction of transcription and translation.

Fig. 2 The coding sequence for E protein

The sequence is as reported by Irie et al., 1989 for DEN-2 (New Guinea-C strain).

Fig. 3. Construction of expression plasmids with N- and C-terminal deletions.

The plasmid containing E coding sequence (pKT 2.4) was digested with PvuII, Tth111I, ScaI, SphI, followed by digestion with EcoRI in all cases to obtain DNA fragments containing progressingly longer deletions of E coding sequences. The fragments were blunted, and inserted into pOTS expression plasmid (Fig. 1) at the blunt-ended BamHI site. The clones are

referred to as pTT10, pTT15, pTT20, pTT25, respectively. The internal deletion mutant was constructed by digesting Tth111I-pOTS expression plasmid with BamHI and ligated to exclude the region between the two BamHI sites (pTT30).

Fig. 4 Coding sequence of E containing the epitope of 3H5 antibody.

The coding sequence of E polypeptide between two BamHI sites (amino acid residues 254-423 (Irie et al., 1989) is shown. Primers used for PCR are underlined. The dotted box encodes the putative epitope of 3H5.

Fig. 5

The hydropathy plot using the hydrophobicity values of Kyte and Doolittle (1982). The darkened area of hydrophilic domain corresponds to 3H5 epitope.

Fig. 6 Western blot analysis of E polypeptides expressed from deletion constructs.

The E coding sequence between PvuII and EcoRI was cloned into pOTS expression plasmid (pTT10). The deletion constructs in the N-terminal and C-terminal (flanked by two BamHI sites) were made as described by cutting with various restriction enzymes such as Tth111I (pTT15), ScaI (pTT20), SphI (pTT25), or NcoI (pTT35), followed by EcoRI. The polypeptides expressed from E coli N5151 transformed by plasmids, pTT10, pTT15, pTT20, pTT25, pTT30, pTT35 are shown in lanes 1-6, respectively. Lane M, molecular weight markers. Lane U, uninduced E. coli cells transformed by expression plasmid. Lane C, control extract from DEN-2-infected C6/36 cells. In A, hyperimmune mouse ascites fluid, and in B 3H5 were used for immunoblots.

Fig. 7 Western blot analysis of E polypeptides expressed from deletion constructs obtained by PCR.

The PCR amplified DNA using primers 1 and 2, 1 and 3, 1 and 4, 1 and 5, and 1 and 6 were isolated as described in Fig. 5, and cloned into pTT15 expression plasmid at the blunt-ended BamHI sites. The lysates of E. coli transformants of these deletion constructs were analyzed by Western blot using HMAF (A), and 3H5 (B). Lane M, molecular weight markers; Lane C, control extract from DEN-2 infected C6/36 cells; Lanes 1-7: E. coli

lysate from cells transformed by expression plasmid pTT30, pTT40, pTT45, pTT50, pTT55, pTT60, pTT15, respectively.

Fig. 8 Enzyme-linked immunosorbent assay (ELISA) for synthetic peptide corresponding to the 3H5 epitope

Reactivity of 3H5 with the synthetic peptide having an extra cysteine at the N-terminus was measured by a modification of the ELISA method as described by Engvall (1980). Microtiter plates (Corning, New York) were coated with 50 µl of synthetic peptide (20 µg/ml) in 50 mM carbonate/bicarbonate buffer (pH 9.6) overnight at 37 C. The antigen-coated wells were washed three times with 0.85% NaCl containing 0.05% Tween 20. After washing, the plates were incubated with blocking solution containing 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-Tween 20 (0.05%) (100 µl/well) for one hour at 37 C. Plates were then incubated sequentially with 50 µl/well of appropriate dilution of antiserum in PBS-Tween 20 containing 1% BSA, goat anti-rabbit antibody conjugated to horseraddish peroxidase (Hyclone, Logan, Utah), H_2O_2 as the substrate, and the orthophenylenediamine as the chromogen (Sigma). Plates were washed between incubations with 0.05% Tween 20 in 0.85% NaCl. The final enzyme reaction was stopped with 2N H_2SO_4, and the color change was measured at 492 nm on a Titertek Multiscan plate reader (Flow Laboratories, McLean Virginia). **A.** The specific binding of the synthetic peptide with 3H5 antibody was tested by ELISA. 3E9 is a monoclonal antibody against the non-structural protein of DEN-2 NS1. A16 is a monoclonal antibody against one of HSV-2 glycoproteins. **B.** Specific reactivity of anti-peptide antisera against the synthetic peptide was measured by ELISA. The microtiter wells were coated with synthetic peptide, and then reacted with different dilutions of the rabbit anti-peptide antisera .

Fig. 9 Strategy for generating transcripts containing the same polarity and 3'-terminal sequences as DEN-2 RNA in vitro

A DNA fragment of 1.34 kilobase pairs containing the 3'-terminal sequences of DEN-2 RNA was obtained by PCR and digested with KpnI as described in MATERIALS AND METHODS (see also Irie et al., 1989). This fragment was cloned into pGEM 7Zf$^+$ vector (Promega Biotec, Madison, WI) which was treated sequentially with XbaI and PolkI to make it blunt-end, and then with KpnI. The clones were screened by the regeneration of XbaI site and finally by DNA sequence analysis . Subclones from the 1.34 kb fragment which have identical 3'-terminus at the XbaI site, but are shorter from the 5'-end were prepared by digesting the parent clone with SacI and HindIII for various time intervals, followed by S1 nuclease treatment (Henikoff, 1984). The end point of a deletion was determined by sequence analysis.

Fig. 10 Analysis of RNAse A-protected fragments generated from the 3'-terminal transcripts

A. Two cDNA clones having 3'-terminal sequences of 1339 bp and 292 bp as inserts were sequentially treated with XbaI, and S1 nuclease. They were then used for the in vitro transcription catalyzed by SP6 RNA polymerase.as described under MATERIALS AND METHODS An aliquot of the labeled transcript (5×10^4 - 1×10^5 cpm) was digested with RNase A (3 µg/50 µl reaction mixture) in a buffer containing 0.01 x SSC (lanes 1-3, or 2 x SSC (lanes 4-6)and the samples were prepared for analysis of the protected fragments by 7M urea/polyacrylamide gel electrophoresis and autoradiography as described under MATERIALS AND METHODS. Lanes 1-6: transcript from 1339 bp cDNA insert (lanes 1-3), or 292 bp insert (lanes 4-6), incubated in a buffer (50 µl), containing 0.01 x SSC (1 x = 0.015M Na-citrate and 0.15M NaCl, pH 7.0) (lanes 2 and 5), or 2 x SSC (lanes 3 and 6), and either without RNase A (lanes 1 and 4), or with 0.2 µg RNase A (lanes 2, 3, 5, and 6) at 37^o for 60 min. The sizes of the fragments were determined from a ladder of 3'-labeled E. coli tRNA fragments generated by alkaline hydrolysis (not shown). The sizes of the three fragments shown by arrows are estimated to be 23, 28, and 33 nt. **B.** The transcript containing 1339 nucleotides of 3'-terminal sequence of DEN-2 RNA (lanes 1 and 2), and a control transcript from a cDNA clone corresponding to nt 1-550 from the 5'-terminus of

DEN-2 RNA (lanes 3 and 4) were either incubated in buffer with no RNase A (lanes 1 and 3), or digested with RNase A (lanes 2 and 4) under the same conditions as in Fig. 10A, lanes 3 and 6 The samples were analyzed by electrophoresis and autoradiography as described under MATERIALS AND METHODS. C. The labeled transcript was incubated in a buffer containing either 0.01 X SSC (lanes 1 and 3), or 2 X SSC (lanes 2 and 4). RNase A (0.2 μg) was added to samples (lanes 1 and 2), or RNase T1 (1 unit) (lanes 3 and 4). The samples were analyzed by electrophoresis and autoradiography as described under MATERIALS AND METHODS.

Fig. 11 Mapping of the RNase A-resistant fragments by annealing specific synthetic oligodeoxynucleotides, followed by RNase H digestion.

The transcripts containing 1339 (A), and 292 (B) nt of 3'-terminal DEN-2 sequences were digested with RNAse A under the same conditions as in Fig. 10A, lanes 3 and 6. The digests were treated with phenol:$CHCl_3$, and the labeled RNA was precipitated with ethanol in the presence of E. coli tRNA (0.5 μg) added as a carrier. Each of the digests was divided into aliquots, and hybridized with synthetic oligodeoxynucleotides complementary to nucleotides 1-96 of DEN-2 RNA as described under MATERIALS AND METHODS. The annealed mixture was then digested with RNase H (one unit) for 60 min at 37° C. The reaction was stopped by the addition of a mixture containing formamide (80%) and bromophenol blue dye. The products of RNase H digestion were analyzed by electrophoresis and autoradiography. Lanes 1-7:, an aliquot of control RNase A digest in the absence (lane 1), or presence of RNase H (lanes 2-7) In lanes 3-6, RNase H was added subsequent to hybridization with oligodeoxynucleotides complementary to nt 1-24 (oligomer 1 in lane 3), 25-48 (oligomer 2 in lane 4), 49-72 (oligomer 3 in lane 5), and 73-96 (oligomer 4 in lane 6) of the 3'-terminal sequence of DEN-2 RNA; In lane 7, mixture of all four oligodeoxynucleotides were annealed prior to RNase H digestion.

Fig. 12 A model for the putative RNase A-sensitive sites in the previously proposed secondary structure for DEN-2 RNA at 3'-end

A. The 3'-terminal sequences of DEN-2 RNA has a potential to form a stable and a highly conserved secondary structure (Hahn et al., 1988; Deubel et al., 1988), which was originally proposed for yellow fever virus RNA (Rice et al., 1985). The numbers refer to the nt position from the 3'-terminus of DEN-2 RNA. Sequences are identical for DEN-2 RNA of New Guinea, Jamaica strains and Puerto Rico-S1 (PRS1) vaccine strains, except at position 42, which is a guanosine for PRS1 strain (Hahn et al., 1988; Dubel et al., 1988; Irie et al., 1989). The large arrows indicate the possible sites of cleavage by RNase A that would give rise to the protected fragments obtained as shown in Fig. 10. The small arrows indicate the putative cleavage sites of RNase T1 that would give rise to the protected fragments obtained as shown in Fig. 2C. The base paired region shown by dotted lines is sensitive to RNase A and RNase T1. B. Structures of possible substrates for RNase H digestion. The RNA:DNA hybrids that would be formed in the annealing of RNase A-resistant fragments with synthetic oligodeoxynucleotides # 2 and 3 are shown.

Table I

Neutralization of Dengue-2 Virus by Rabbit Anti-Peptide Serum[a]

Antiserum Dilution[b]	Mean Percent Plaque Reduction[c] Experiment 1	Experiment 2
1:10	95.6%	90.2%
1:20	79.6%	76.7%
1:40	47.1%	50.5%
1:80	N.D.[d]	29.8%

a) Rabbit anti-peptide serum obtained from rabbits immunized with dengue-2 E protein specific, MoAb 3H5-reactive peptide

b) Serum dilutions made in Hank's balanced salt solution containing 2% fetal bovine serum

c) Mean Percent Plaque Reduction calculated from the average of duplicate titrations as:
1.0-(plaques with immune serum/plaques with nonimmune serum), expressed as a percent

d) Not done

Fig. 1

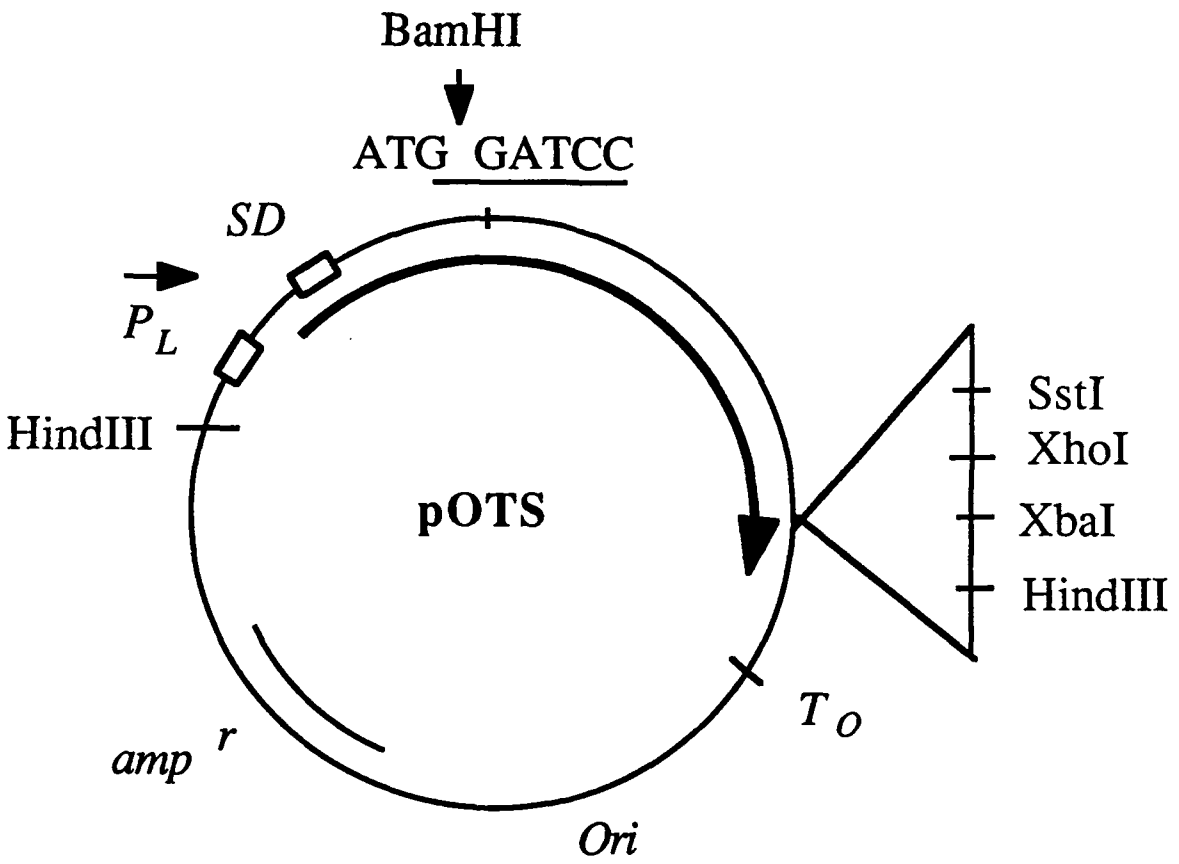

BamHI
ATG GATCC
SD
P_L
HindIII
pOTS
SstI
XhoI
XbaI
HindIII
T_O
amp r
Ori

Fig. 2

Coding Sequence of DEN-2 E Protein

MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIETEAKQPATLRKYCIEAKLTNTTTDSRCPTQGEP

SLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFTCKKNMKGKVVQPENLEYTIVITPHSGEEHAVGNDTGKHGK

EIKITPQSSITEAELTGYGTVTMECSPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF

KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYSMCTGKFKVVKEIAETQHGTI

VIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQ

MIETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWIMKILIGVIITWIGMNSRSTSLSVSLV

LVGVVTLYLGVMVQA

Fig. 3

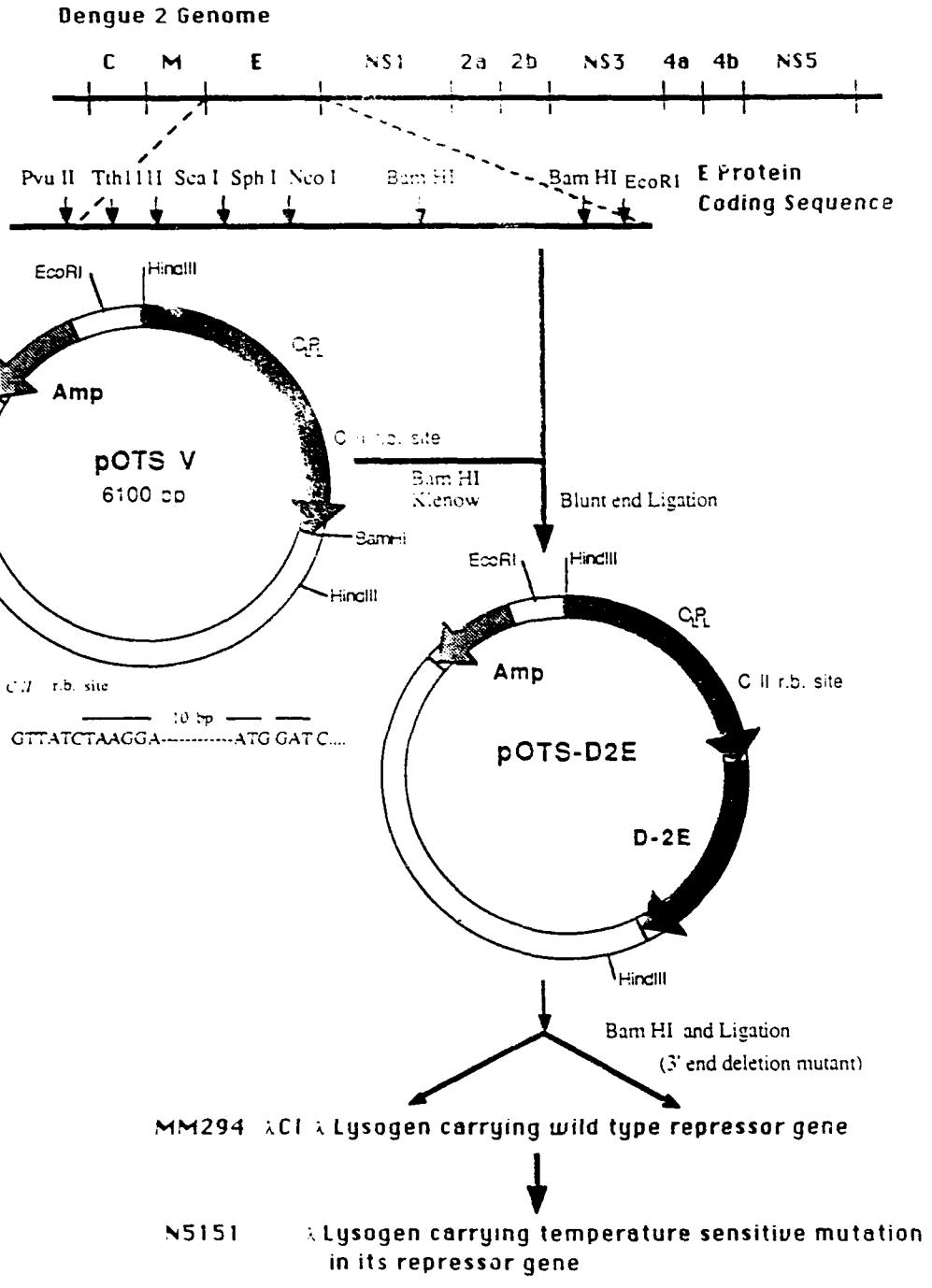

Dengue 2 Genome
C M E NS1 2a 2b NS3 4a 4b NS5
Pvu II Tth111I Sca I Sph I Nco I Bam HI Bam HI EcoRI
E Protein Coding Sequence
EcoRI
HindIII
Amp
pOTS V
6100 bp
Bam HI Klenow
Blunt end Ligation
BamHI
HindIII
C II r.b. site
10 bp
GTTATCTAAGGA----------ATG GAT C....
EcoRI
HindIII
Amp
C II r.b. site
pOTS-D2E
D-2E
HindIII
Bam HI and Ligation
(3' end deletion mutant)
MM294 λCI λ Lysogen carrying wild type repressor gene
N5151 λ Lysogen carrying temperature sensitive mutation in its repressor gene

Fig. 4

Deletion Strategy of Mapping the Epitope by Polymerase Chain Reaction

a.a.#

Primer # 1

GGA TCC CAA GAA GGG GCC ATG CAC ACA GCA CTC ACA GGG GCC ACA GAA ATC CAG ATG TCA

254 – G S Q E G A M H T A L T G A T E I Q M S

TCA GGA AAC TTA CTG TTC ACA GGA CAT CTC AAG TGC AGG CTG AGG ATG GAC AAA CTA CAG

274 – S G N L L F T G H L K C R L R M D K L Q

Primer # 2

CTC AAA GGA ATG TCA TAC TCT ATG TGC ACA GGA AAG TTT AAA GTT GTG AAG GAA ATA GCA

294 – L K G M S Y S M C T G K F K V V K E I A

GAA ACA CAA CAT GGA ACA ATA GTT ATC AGA GTA CAA TAT GAA GGG GAC GGT TCT CCA TGT

314 – E T Q H G T I V I R V Q Y E G D G S P C

Primer # 3...

AAG ATC CCT TTT GAG ATA ATG GAT TTG GAA AAA AGA CAT GTT TTA GGT CGC CTG ATT ACA

334 – K I P F E I M D L E K R H V L G R L I T

....Primer # 3

GTC AAC CCA ATC GTA ACA GAA AAA GAT AGC CCA GTC AAC ATA GAA GCA GAA CCT CCA TTC

354 – V N P I V T E K D S P V N I E A E P P F

Primer # 4

GGA GAC AGC TAC ATC ATC ATA GGA GTA GAG CCG GGA CAA TTG AAG CTC AAC TGG TTT AAG

374 – G D S Y I I I G V E P G Q L K L N W F K

Primer # 5

AAA GGA AGT TCT ATC GGC CAA ATG ATT GAG ACA ACA ATG AGG GGA GCG AAG AGA ATG GCC

394 – K G S S I G Q M I E T T M R G A K R M A

Primer # 6

ATT TTA GGT GAC ACA GCT TGG GAT TTT GGA TCC

414 – I L G D T A W D F G S

Fig. 5

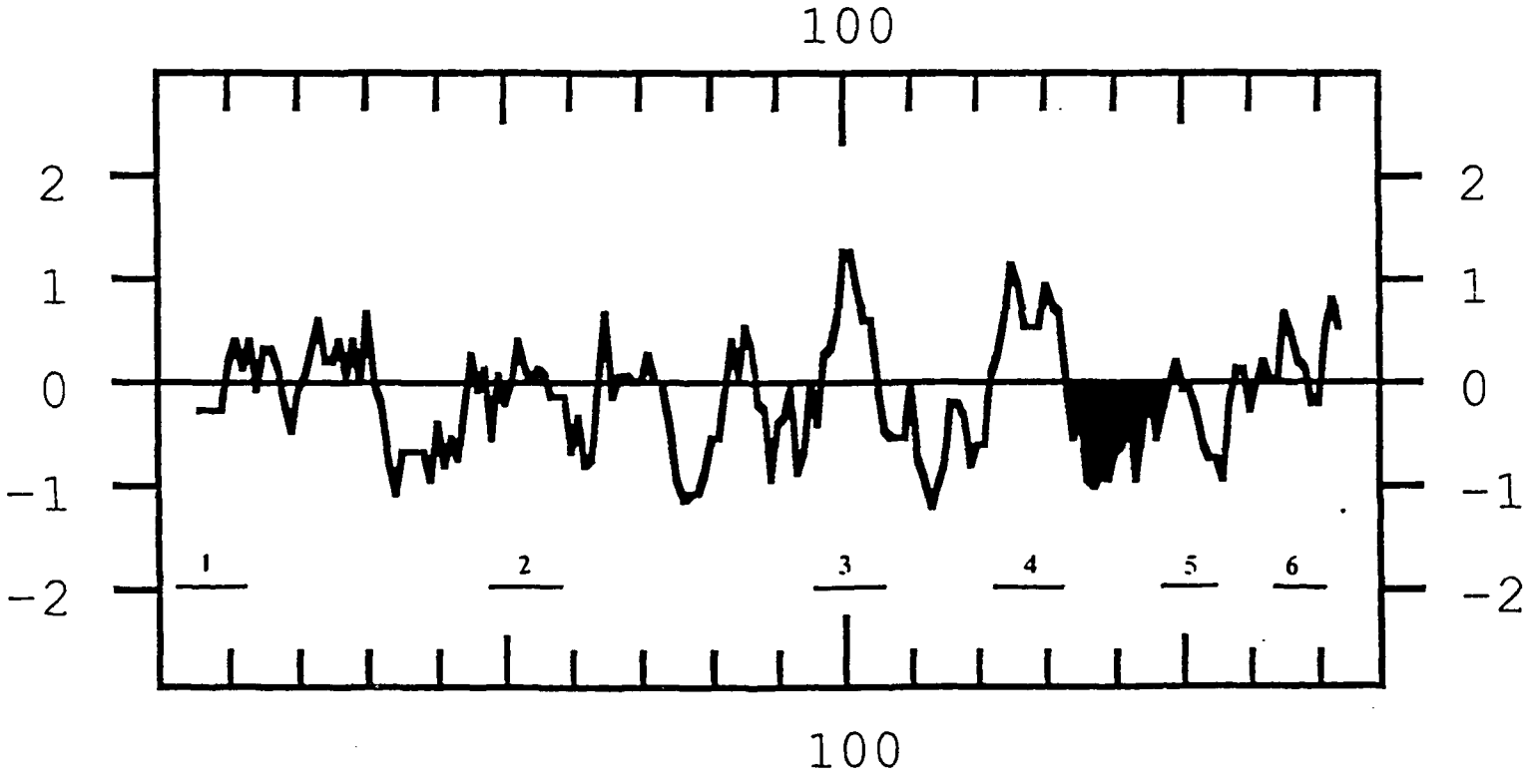
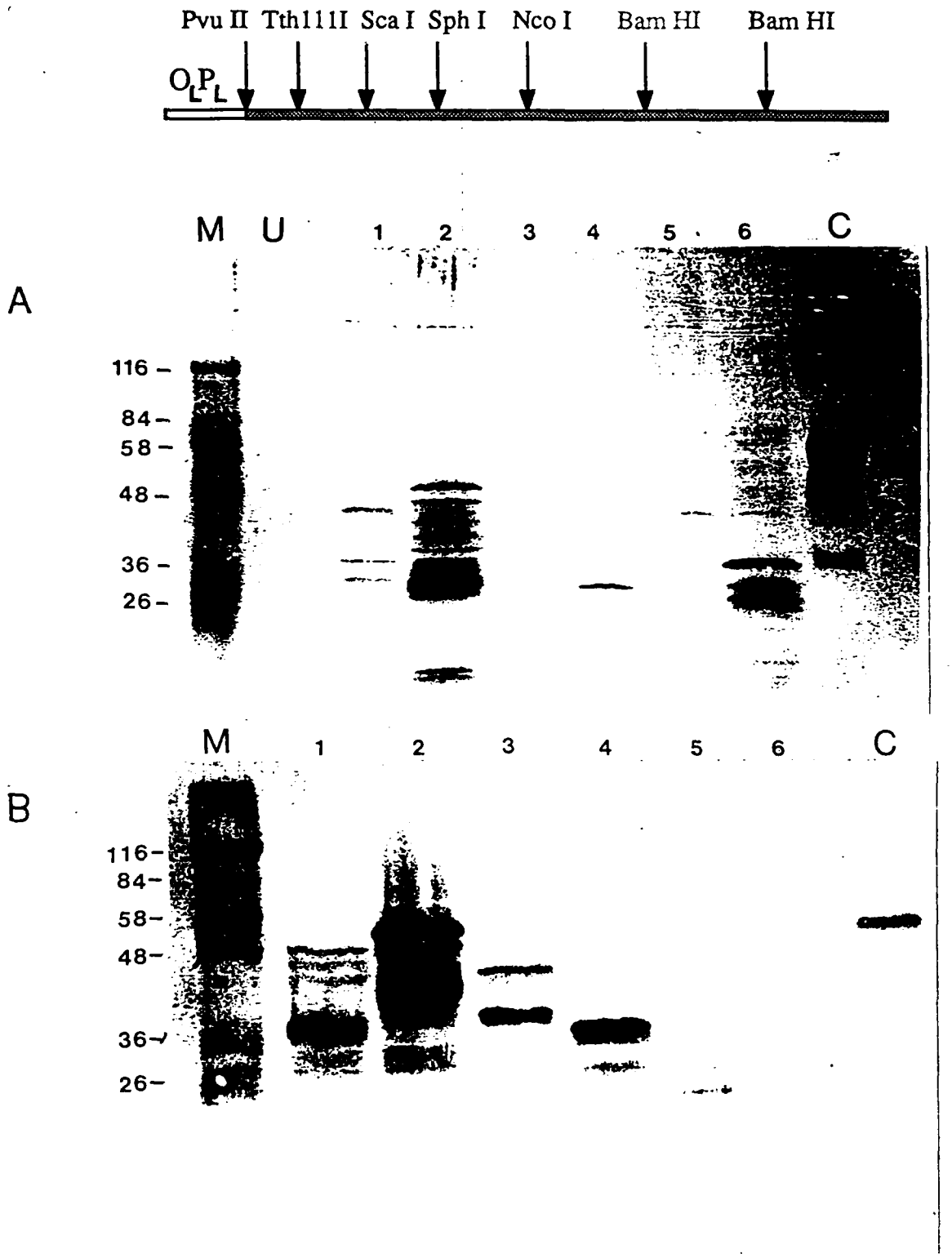


Fig. 6



Western blot analysis of N-terminal and C-terminal deletion mutants

A. Hyperimmune mouse ascites fluid against DEN-2 (HMAF,
B. Monoclonal antibody 3H5

Fig. 7

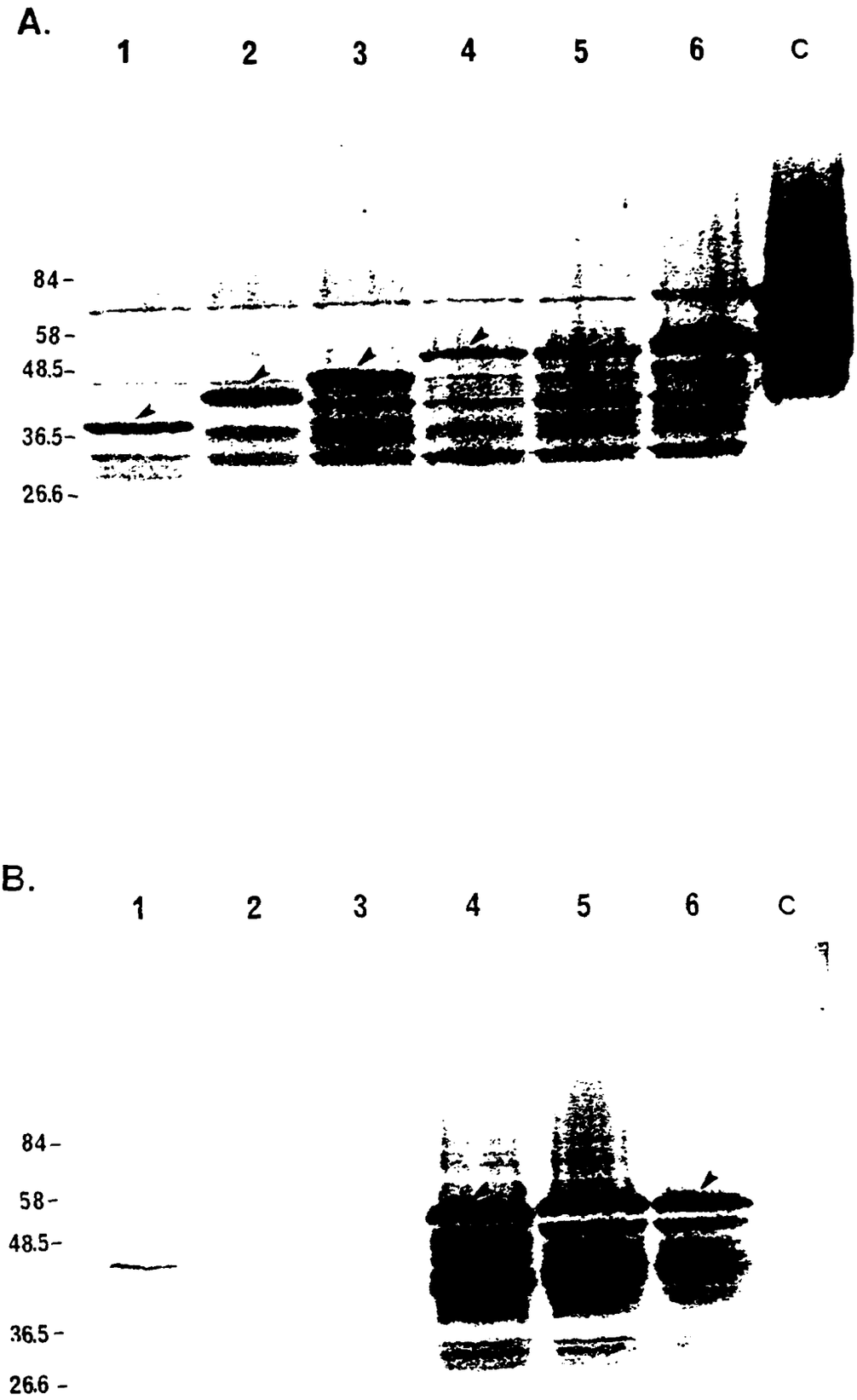


Fig. 8

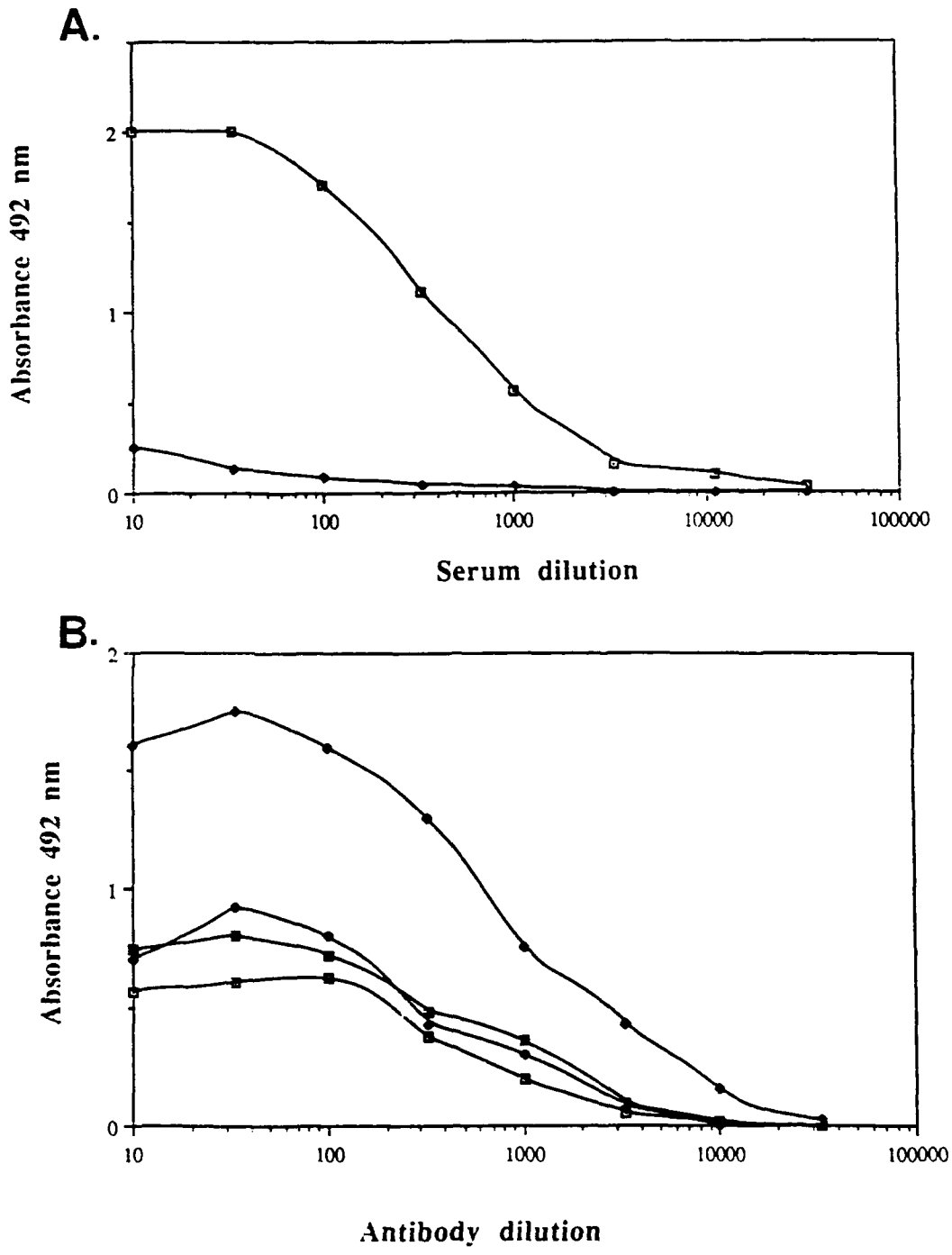

A.
Absorbance 492 nm
2
1
0
10
100
1000
10000
100000
Serum dilution
B.
Absorbance 492 nm
2
1
0
10
100
1000
10000
100000
Antibody dilution

Fig. 9

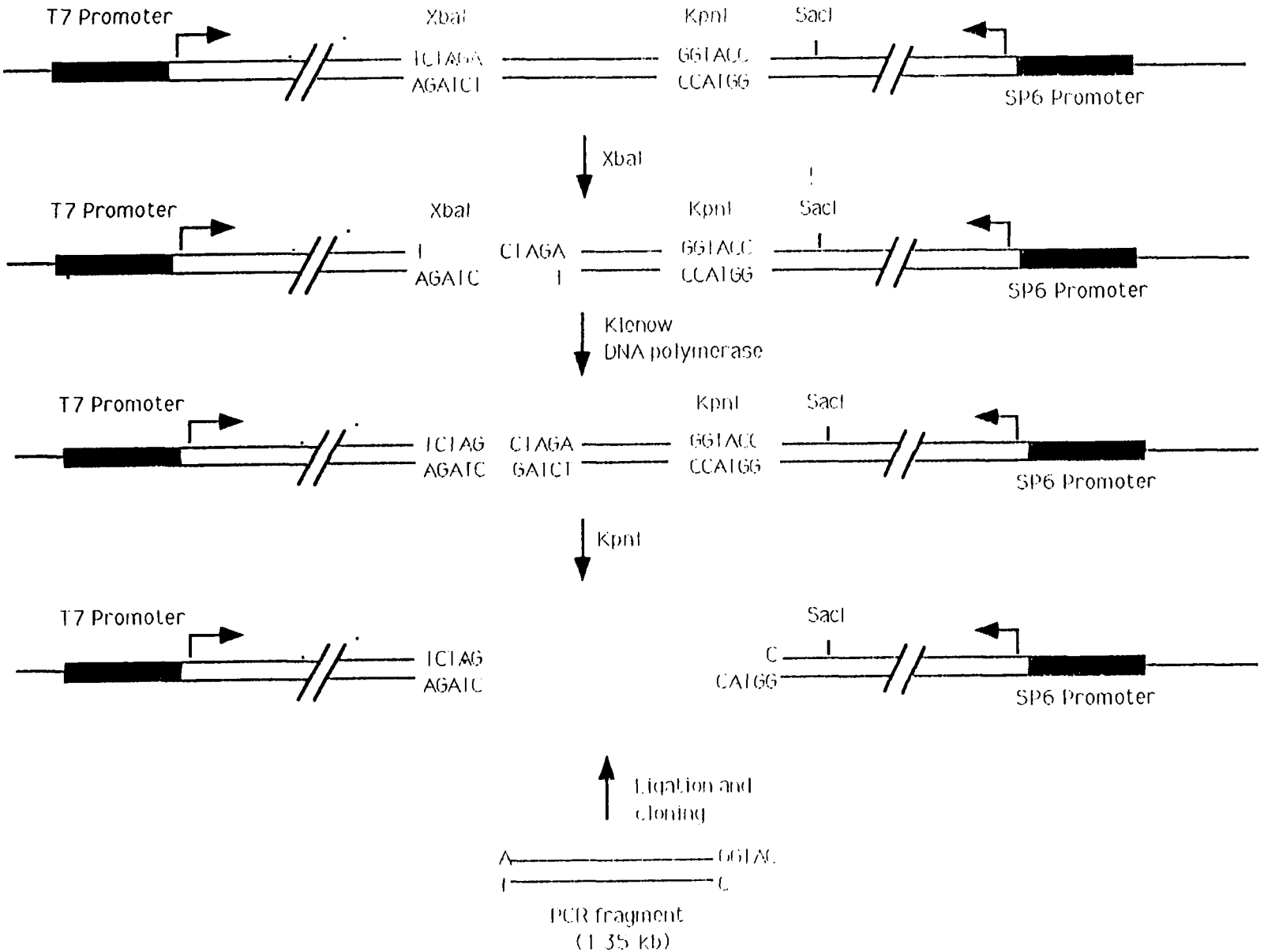

T7 Promoter
XbaI
KpnI
SacI
TCTAGA
AGATCT
GGTACC
CCATGG
SP6 Promoter
XbaI
T7 Promoter
XbaI
KpnI
SacI
CTAGA
GGTACC
CCATGG
SP6 Promoter
Klenow
DNA polymerase
T7 Promoter
KpnI
SacI
TCTAG
AGATC
CTAGA
GATCT
GGTACC
CCATGG
SP6 Promoter
KpnI
T7 Promoter
SacI
TCTAG
AGATC
C
CATGG
SP6 Promoter
Ligation and
cloning
GGTAC
C
PCR fragment
(1.35 kb)

Fig. 10

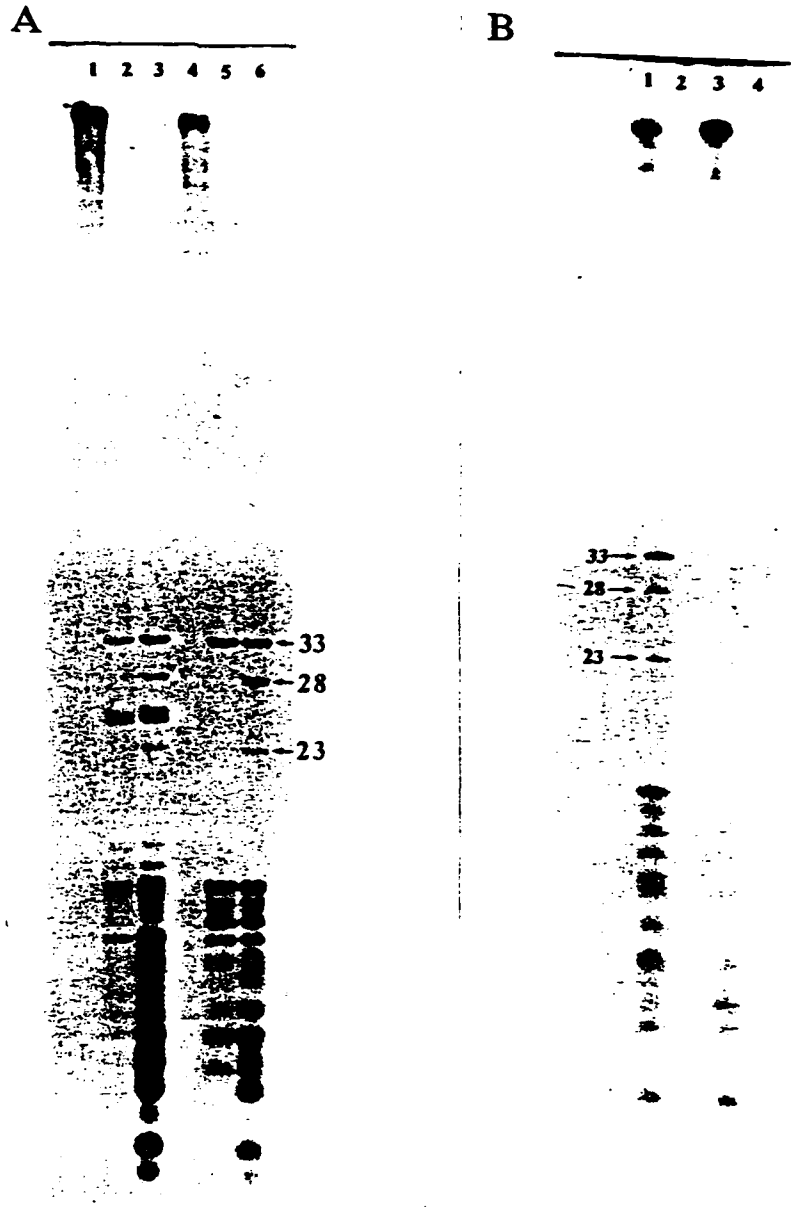


Fig. 10C

Fig. 11

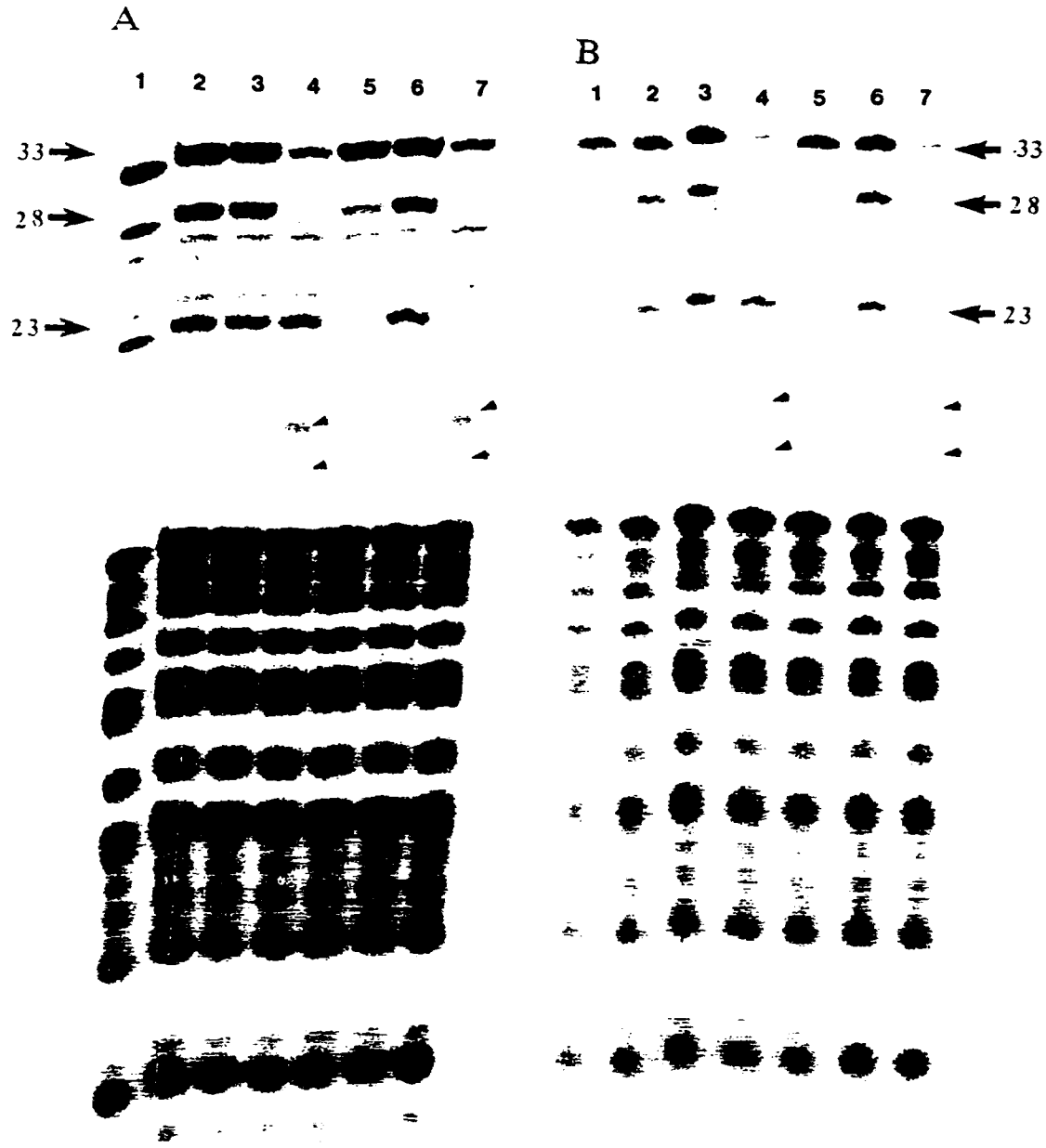


Fig. 12A

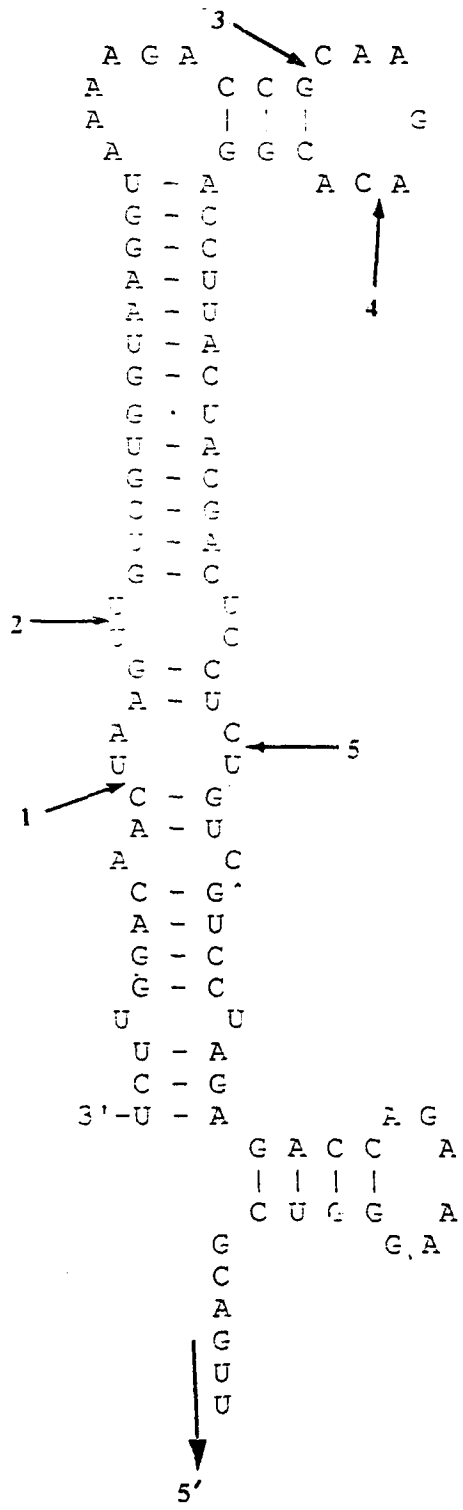
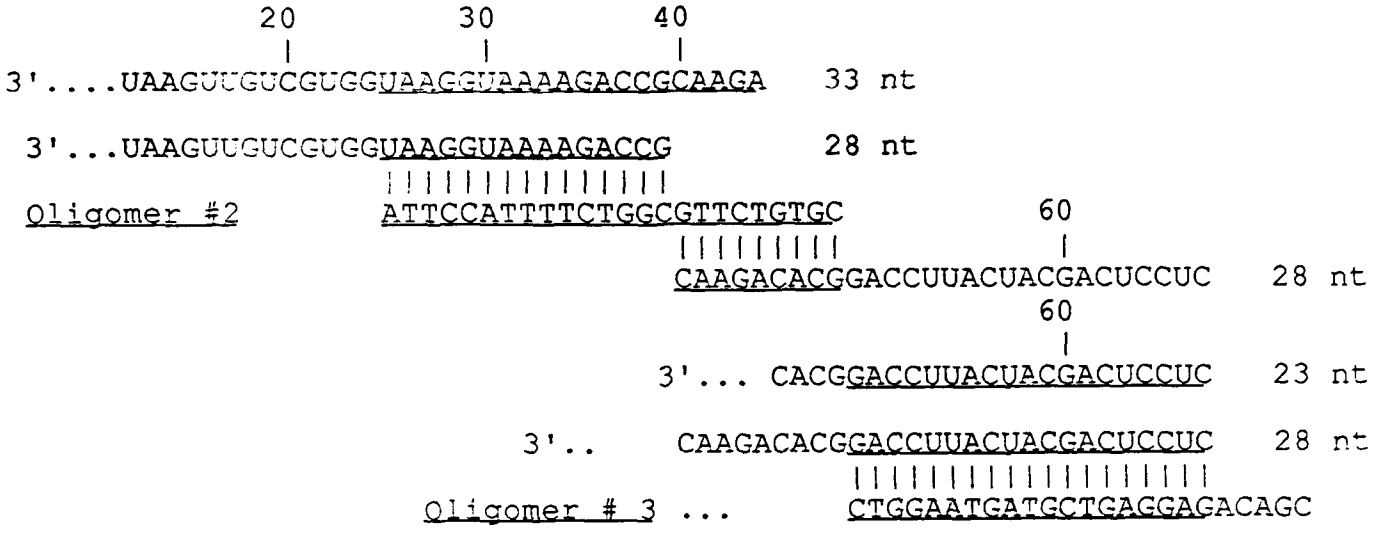


Fig. 12B


20
30
40
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3'...UAAGUUGUCGUGGUAAGGUAAAAGACCG 28 nt
Oligomer #2
ATTCCATTTTCTGGCGTTCTGTGC
60
CAAGACACGGACCUUACUACGACUCCUC 28 nt
60
3'... CACGGACCUUACUACGACUCCUC 23 nt
3'.. CAAGACACGGACCUUACUACGACUCCUC 28 nt
Oligomer # 3 ... CTGGAATGATGCTGAGGAGACAGC

6.0

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Raghuran Kalluri	Graduate Student	50%	9/15/88 - 3/5/89	(5 1/2 mos.)
Parmathi Mohan	Fellow	100%	10/2/88 - 3/21/89	(5 1/2 mos.)
Tha. Trirawatanapong	Graduate Student	50%	9/15/88 - 9/14/89	(12 mos.)
Yifan Xu	Research Technician	100%	2/5/89 - 6/25/89	(5 mos.)
Luwen Zhang	Graduate Student	50%	1/22/89 - 9/14/89	(7 1/2 mos.)